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ROY WALDO MINER

NUTRITIONAL FACTORS IN THERMOPHILY: A COMPARATIVE STUDY OF BACILLI AND *EUGLENA*

BY

HERMAN BAKER, S. H. HUTNER, AND H. SOBOTKA

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The biochemical prerequisites for thermophily (here defined as growth above 55° C.) have attracted intense interest because of the insights offered into the determinants of the stability of protoplasm, particularly of proteins and enzymes. Furthermore, as the nutrition of thermophilic bacilli (henceforth denoted simply as "thermophiles") is becoming known in some detail, thermophiles are emerging as tools for uncovering many metabolic pathways hitherto almost completely inaccessible to analysis by the microbial tools which have been so successful with other systems; *e.g.*, B vitamins, nucleic acid components, and amino acids. This paper describes the development of chemically defined culture media for representatives of several species of thermophiles including many strains of *Bacillus stearothermophilus*. Several of these strains have been grown here in chemically-defined media at 80° C.

Since permeability emerged as one of the important—perhaps a decisive—factor in thermophily, the investigation was broadened to include a study of *Euglena gracilis*, strains of which fell into two sharply distinct groups in respect to temperature optima and the correlated use of organic nutrients. Strains of *E. gracilis* that live at the higher temperatures utilize a much wider array of substrates than do the "low temperature" forms.

As media were improved, the rapidity of growth and the freedom from contamination revealed possibilities for assays for B vitamins and for B vitamins and for thermolabile materials. The increased solubility of lipids at these high incubation temperatures likewise opened avenues of attack on the nutritional biochemistry and histological distribution of lipids. We thought it worthwhile, therefore, to describe in some detail the experiments that were designed to evolve techniques for making lipids nutritionally effective to bacteria. This is a matter of some moment, because, for lack of microbiological assay methods, knowledge of the mode of action of such lipids as the fat-soluble vitamins has lagged far behind corresponding knowledge of the B vitamins. In this respect, the present investigation broadened beyond the original aim of understanding the metabolic basis of thermophily.

Part I. Thermophilic Bacilli

HISTORY AND DESCRIPTION OF STRAINS

Nearly all previous work has been adequately covered in two recent

reviews.[1,2] The weight of evidence is that thermophiles have an exceptionally rapid metabolic turnover, marked by a narrow margin between synthesis of essential metabolites and their heat-accelerated destruction. This "dynamic" view of thermophily is advocated by Allen.[1] Concurrently, there is increasing evidence that many, perhaps most, of the enzymes of thermophiles are more resistant to heat denaturation, or for other reasons have higher temperature optima than do their mesophilic (*i.e.*, temperature optima *ca.* 37° C.) counterparts.

The taxonomy of the aerobic spore-forming bacilli has been treated critically and comprehensively.[3] The thermophiles fall into two well-defined species: (1) *Bacillus coagulans*, the majority of whose strains grow between 33° and 60° C., none above 60° C.; and (2) *Bacillus stearothermophilus*, growing between 37° and 65° C., with many growing above 70° C. Doctor Ruth E. Gordon of Rutgers University, New Brunswick, N. J., kindly sent us her collection of authentic strains of *B. stearothermophilus*. Other authentic strains were obtained from the National Canners Association, Washington, D. C., through the courtesy of Doctor E. J. Cameron.

The minimal nutritional requirements of a few strains of *B. stearothermophilus* had been identified in several laboratories. All 12 strains of *B. stearothermophilus* studied by Cleverdon *et al.*,[4] responded to thiamine, nicotinic acid, and biotin. These investigators noted that comparatively high concentrations of these vitamins were required. They did not determine whether these vitamins were absolute requirements for all their strains. Campbell and Williams[5] carried this analysis further: essential amino acids and vitamins were studied as a function of temperature up to 55° C. Their findings for *B. stearothermophilus* are summarized in TABLE 1. One notes that while, in general, the complexity of requirements increases with temperature, this is not always so.

CULTURE METHODS

1. *Apparatus*

The technique used, in the main, was that previously described but, because of the higher incubation temperatures (65° to 80° C.), modifications were necessary. Experimental media were distributed in either 10- or 35-ml. borosilicate flasks provided with aluminum caps. The 10-ml. size was used for large-scale surveys; the 35-ml. size for detailed work, especially at higher temperatures. The solubility of O_2 diminishes rapidly with increase in temperature; for example, O_2 is only half as soluble at 60° C. as at 30° C. Therefore shallower layers of media had to be employed. Furthermore, evaporation of media at the higher temperatures becomes a serious problem. In turn, the heat of evaporation caused large fluctuations in temperature when the incubators were opened for in-

spection of cultures. Stability of temperature and slowing of evaporation had been achieved by setting the flasks in Pyrex baking dishes containing a layer of water, making a partly sealed unit by inverting a duplicate dish over the first and sealing the joint with transparent cellulose tape. At higher temperatures, because of the increased evaporation, the flasks were set in a deeper layer of water in the trays. This tended to make the flasks easy to upset. The 35-ml. borosilicate flasks used later (made for this purpose by the Kimble Glass Company, Vineland, N. J.) had extra-heavy walls and a broad base, and so were less prone to tip over.

For work above 60° C., special ovens had to be designed in which evaporation could be held to a minimum, power consumption kept within the limits of the usual laboratory electrical supply, and temperature fluctuations held to a minimum. The main source of temperature fluctuations proved to be the vaporization of water required to replace the water vapor lost upon opening the incubators. Another difficulty was the

TABLE 1

EFFECT OF INCUBATION TEMPERATURE ON THE NUTRITIONAL REQUIREMENTS OF *B. STEAROTHERMOPHILUS*
After Campbell and Williams[5]

*Strain**	*36° C.*	*45° C.*	*55° C.*
1356	No growth	Leucine, thiamine, nicotinic acid, biotin	Same as 45° C.
13736	No growth	Glutamate, histidine, methionine, leucine, biotin	Same as 45° C. + B_6
1503	No growth	Valine, nicotinic acid, biotin	Same as 45° C.
3084	Biotin, folic	Same as 36° C.	Same as 36° C. + thiamine
3656	No growth	Histidine, nicotinic acid, biotin	Same as 45° C.
3690	Methionine, leucine, thiamine, nicotinic, biotin, folic	Leucine and nicotinic unnecessary	Same as 45° C.
4259	Biotin, folic	Same as 36° C. + methionine, histidine, folic	Same as 45° C.
5149-5	Methionine, thiamine, biotin, folic	Biotin, folic	Same as 45° C.

*National Canners Association designation.

accelerated corrosion resulting from saturation humidities at higher temperatures. A completely satisfactory incubator is not yet available but, in cooperation with the Electric Hotpack Company of Phiiadelphia, Pa., improved incubators are being developed. The current model now in use for work at *ca.* 80° C. includes these features: (1) stainless steel construction throughout; (2) glass observation port together with glass inner door; (3) low-gradient heating elements; and (4) thick glass-wool insulation and extra-heavy rubber gaskets on doors.

These details are mentioned because previous work on thermophiles was hindered by the unavailability of suitable incubators. Other workers have used water baths but, at elevated temperatures, evaporation from the baths made temperature control poor, and, to conserve the limited space in the baths, cultures usually were placed in upright test tubes. This reduced the availability of O_2 to a point where forced aeration or agitation was necessary for satisfactory growth. Since the present study includes a survey of the nutritional requirements of over 160 isolates of thermophiles, our technique was designed to avoid complications of apparatus.

Growth was measured in optical density (O.D.) units as determined with a Welch Densichron. An O.D. of 1.0 equaled 0.5 to 0.55 g./liter dried washed bacteria.

2. *Basal Media for Screening*

For preliminary determination of nutritional requirements, the following basal medium was used (TABLE 2). Cultures were maintained on beef extract (several brands), 0.5 g.; "Polypeptone" (Baltimore Biological Laboratory), 0.5 g.; agar, 2.0 g.; distilled water, to 100 ml. To maintain solidity at high temperatures, the agar was increased to 3.0 per cent. Stock cultures were incubated at 55° C. for 24 hours and stored at 4 to 7° C., and transferred every four to six weeks. Transfers at longer intervals did not affect viability

RESULTS

1. *General Pattern of Exogenous Growth Requirements in* B. stearothermophilus

All of the 60 strains that were screened required biotin, and most required, in addition, thiamine and nicotinic acid. Most strains had still additional requirements. Only a few of these strains have been studied in detail throughout the temperature range 37° C. to 75° C. One among these, No. 39 (designation of Gordon) did not grow below 45° C. At 55° C. it required only biotin. At 75° C., in experiments carried out in cooperation with Doctor Sidney Saperstein, histidine was required. The

TABLE 2

BASAL MEDIUM FOR SCREENING

Versen-Ol	0.02 g.	Ca (as Cl^-)	2.0 mg.
K glycerophosphate	0.05 g.	Mo (as NH_4 salt)	0.2 mg.
$MgSO_4 \cdot 7H_2O$	0.04 g.	V (as SO_4)	0.01 mg.
Aconitic acid (trans)	0.5 g.	Thiamine HCl	0.2 mg.
Na acetate$\cdot 3H_2O$	0.04 g.	Nicotinic acid	0.2 mg.
Glycerol	2.0 g.	Biotin	4.0 μg.
DL-Asparagine	0.25 g.	Metals "ST"	1.5 ml.

Distilled water to 100 ml.; pH 6.9 to 7.1

Notes:

This medium was made up in double strength and did not precipitate on refrigeration.

1. *"Versen-Ol"*, the trade name for *N*-hydroxyethylethylenediaminetriacetic acid was supplied by Versenes, Inc., Framingham, Mass., as the tri-Na salt. The concentration shown is calculated as the free acid. It replaced the ethylenediaminetetraacetic acid (EDTA) previously used, because of the greater solubility of the Mg and Ca complexes.

2. *"Metals ST"*: 1 ml. =

EDTA	2.5 mg.	Mn	0.6 mg.
Zn	4.0 mg.	Ca	0.05 mg.
Fe	0.5 mg.	Co	0.02 mg.

The metals were supplied as sulfates. The metal mix was kept in an amber bottle.

3. *Mo* and *V* were included for completeness. The stock solutions were kept alkaline (pH 8.0 - 8.6) to avoid precipitation.

4. The *pH* was brought to 6.9 to 7.1 with 2-amino(2-aminoethyl) ethanol, also known as *N*-hydroxyethylethylenediamine. It furnishes 2 equivalents of alkali and therefore did not increase osmotic pressure as much as did NaOH or KOH. It caused darkening of alkaline media, and was there replaced by triethanolamine or dihydroxyethylethylenediamine (Carbide and Carbon Chemicals, Inc., New York, N. Y.)

5. *Aconitic acid* served as pH buffer for the acid range. Citrate was not used because many strains metabolized it, thus upsetting the metal balance of media. Aconitic acid was not metabolized. As commercial aconitic acid is prepared from citric acid, a biological product, and some samples are impure (*e.g.*, they contained appreciable amounts of lysine as shown by assay with a lysine-requiring thermophile), tetracarboxybutane was used for exact determination of growth factors requirements. Tetracarboxybutane was autoclaved separately before use to drive off a volatile contaminant.

6. *DL-asparagine* was a synthetic product bought from the H. M. Chemical Co., Santa Monica, Cal. All strains used it. It was preferred to NH_4 salts because there was no danger of toxicity from free ammonia, as observed in alkaline media, and the aspartic acid resulting from deamidation served as a good auxiliary substrate and N-source.

7. *K glycerophosphate* served as PO_4 source. It was preferred to inorganic PO_4 because of the greater solubility of the Ca and Mg salts. This property was especially desirable in alkaline media. Some strains liberated enough free NH_3 to alkalinize media and so would have caused precipitates in media containing inorganic phosphate.

8. The constituents of the *"metal mix"* had been tested individually and in combination, and the mixture in the levels chosen allowed very dense growth without initial precipitation or toxicity.

9. *Glycerol* was utilized by all strains of *B. stearothermophilus* examined in detail. Gluconate and sucrose were used by nearly all strains of *B. stearothermophilus* and *B. coagulans*.

TABLE 3

ISOLEUCINE-VALINE RELATIONS

Medium contains vitamin B_{12} 0 2 μg %

DL-Isoleucine (mg. per cent)	*DL-Valine (mg. %)*			
	0	*1.0*	*3.0*	*10*
0	0.46	0.28	0.14	0
0.1	1.0	0.44	0.42	0.46
0.3	1.72	0.52	0.6	0.6
1.0	1.5	0.58	0.88	1.88
3.0	0	1.74	1.88	2.56
10	0	0.34	0.56	1.36

combined addition of phenylalanine and glutamate further increased growth. Another strain, No. 194, which grew well in the basal medium at 55° C. and at 75° C., required additional amino acids at 80° C. The 80° C. requirements were satisfied by a mixture of methionine, leucine, and phenylalanine.

Several other strains grew densely at 80° C. in mixtures containing known vitamins and amino acids, but the exact requirements have not yet been determined. The general findings, in agreement with the results at lower temperatures of Campbell and Williams (TABLE 1), was an in-increase in amino acid requirements with increasing temperature.

2. *Vitamin B_{12} -Methionine and Temperature-Bile Relations*

Strain No. 1503 (National Canners Association) which, at 55° C. appeared to have absolute requirements for thiamine, nicotinic acid, and isoleucine, grew densely when methionine was added. Methionine was replaceable by cyanocobalamin. This methionine-B_{12} relation was examined in some detail. A detailed discussion of vitamin B_{12}-methionine relations in this and other microorganisms will appear elsewhere.[7]

The basal medium for this study was the "screening" medium supplemented with DL-valine, 12 mg. per cent; DL-isoleucine, 12 mg. per cent; DL-leucine, 10 mg. per cent; glycine, 10 mg. per cent. Guanylic acid or adenylic acid (2 mg. per cent) further speeded growth. Amino acid interactions will be treated later. The values for half-maximal growth were 0.1 μg. per cent for cyanocobalamin and 1.0 mg. per cent for DL-methionine, a ratio of about 1: 100,000. This is in the same order of magnitude as observed in mutant 113-3 of *Escherichia coli*, which responds interchangeably to cobalamins and methionine.[8] Methionine was

not replaceable by DL-homocystine + betaine or choline, or by cystine. Homocystine (or its thiolactone) was not available for test.

Isoleucine, valine, leucine, and glycine stimulated growth. The isoleucine-valine ratio was fairly critical (TABLE 3.)

On the supposition that fat-soluble materials might be required at higher temperatures, bile was used at this point, to serve both as a source of lipids and of emulsifying agents. As little as 10 mg. per cent of dehydrated ox bile (Difco) was toxic at 55° C. but, in the presence of "complete supplement No. 5" (TABLE 4), strain No. 1503 grew well at both 55° and 65° C. in the presence of as much as 40 mg. per cent ox bile (TABLE 5). A dissection of the components of "complete supplement" revealed that additional DL-isoleucine (5.0 mg. per cent) overcame the toxicity of ox bile at 55° and 65° C. Growth was further stimulated by L-tyrosine (4.0 mg. per cent).

In order to use the highest possible concentration of bile, the isoleucine concentration was raised. This created an imbalance which was corrected by valine and, to a lesser extent, by leucine. Valine, isoleucine, and leucine, used in suitable proportions, allowed dense, rapid growth with as much as 40 mg. per cent ox bile.

3. *Vitamin B_{12}, Folic acid, and P-Aminobenzoic Acid Relations*

In the course of identifying the minimal growth requirements of our collection of *B. stearothermophilus*, several strains were met which responded to folic acid (PGA). The metabolism of folic acid is linked to that of purines, pyrimidines, and certain amino acids, and it was of interest to see whether thermophiles are permeable to these metabolites. One of these, No. 3084, was examined in detail. Since it did not grow

TABLE 4

COMPOSITION OF "COMPLETE SUPPLEMENT NO. 5"

Gelatin hydrolysate	0.4 g.
DL-Methionine	6.0 mg.
DL-Tryptophan	6.0 mg.
Alkali-hydrolyzed yeast nucleic acid (HYNA)	20 mg.
"Vitamin mix No. 10"	1.0 ml.

Notes:

The concentrations indicated are those in the final medium. In practice, the mixture was made up in 20-fold strength, so that it was used at the level of 5.0 ml. of final medium.

Preparation of the *gelatin hydrolysate, hydrolyzed yeast nucleic acid,* and the composition of the "*vitamin mix*" was described elsewhere.[9] The "complete supplement" lacks cystine, which, because of its low solubility, is dissolved in either acid or alkali and added separately.

vigorously in the media devised for No. 1503 (the B_{12}-methionine strain) its inorganic requirements and its stimulation by amino acids was the object of special study, the results of which are embodied in the basal medium in TABLE 6.

The ostensible requirement for folic-acid was also satisfied by p-aminobenzoic acid (PAB) or by a combination of thymine, xanthine, and cyanocobalamin. The concentration of cyanocobalamin required under these conditions seemed unphysiologically high—10 μg. per cent—as judged from the requirement for full growth (*ca.* 0.3 μg. per cent) of strain 1503 (FIGURE 1). Increasing the concentrations of thymine, xanthine, and cobalamin, separately and in combination, did not improve growth. It is evident that a complete by-passing of PAB-folic acid will entail the use of additional metabolites. Substantially the same result was obtained when PAB served as the "folic acid" standard.

Specificity of the PAB-PGA requirement. The equal effectiveness of PAB and PGA indicated that the PAB-PGA requirement reflected a block in the synthesis of PAB. Folinic acid, but not pteroic (both obtained through the courtesy of Doctor H. P. Broquist of the Lederle Laboratories Division of the American Cyanamid Co., Pearl River, N.Y.), was active (*cf.* Lascelles *et al.*[10]).

Much the same picture of the bypassing of the PAB-folic requirement emerged from an analysis of inhibition by sulfanilamide. At high concentrations of sulfanilamide (*ca.* 30 mg. per cent), either folic or folinic acids overcame the inhibition. Pteroic acid was inactive. Inhibition was also released by a combination of thymine, xanthine, and cyanocobalamin

TABLE 5

REVERSAL OF BILE TOXICITY AT 65° C. BY "COMPLETE SUPPLEMENT"

Medium supplemented (mg. %) with DL-methionine 20, DL-valine 12.0, DL-isoleucine 8.0, DL-leucine 5.0, glycine 20, and B_{12} 5 μg				
Conc. bile (mg. %)	*"Complete supplement" (ml. / 100 ml.)*			
	0	*2.5*	*5.0*	*10*
0	0.57	0.87	1.15	1.54
4	0.55	0.77	1.07	1.37
10	0.37	0.71	0.9	1.24
20	0.26	0.62	0.72	1.14
30	0.3	0.73	0.84	1.11
40	0.03	0.05	0.66	1.18
50	0	0	0	0.08

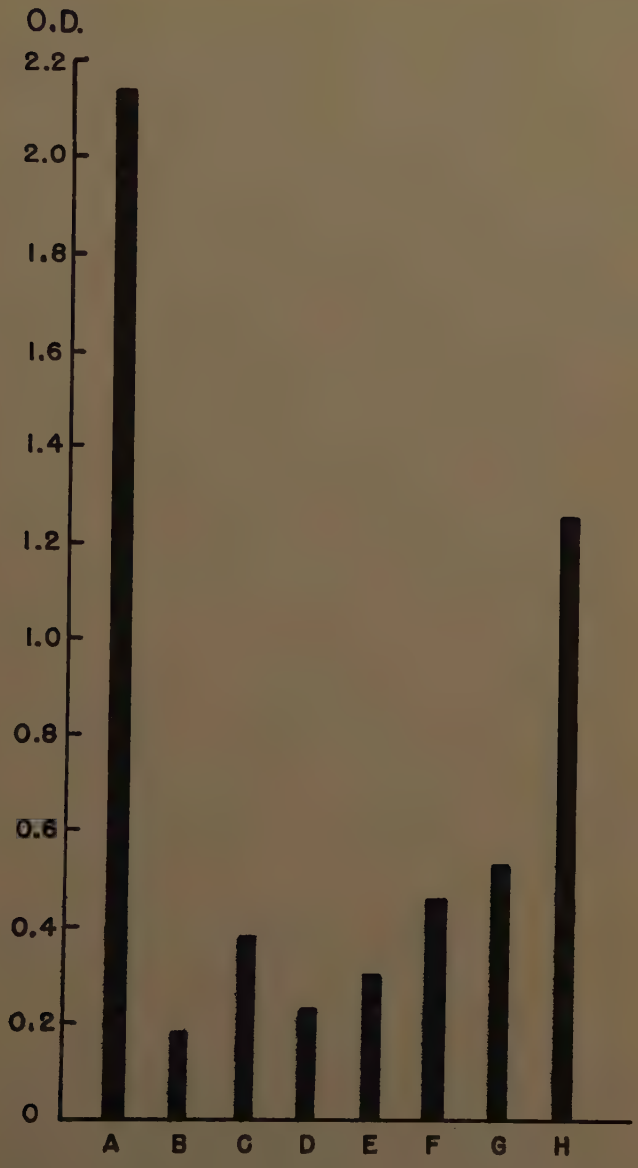

FIGURE 1. Bypassing of pteroylglutamic acid by combined thymine, xanthine, and cyanocobalamin. Basal medium as in TABLE 6. A = PGA; B = thymine; C = cyanocobalamin; D = xanthine; E = thymine + cyanocobalamin; F = thymine + xanthine; G = xanthine + cyanocobalamin; H = thymine + xanthine + cyanocobalamin. Concentrations employed (per 100 ml.): PGA 3.0 μg., thymine 2.0 mg., cyanocobalamin 10 μg., xanthine 2.0 mg.

but, again, this combination allowed *ca.* half the growth achieved with PAB, folic, or folinic acids.

4. *Nutritional Requirements of Other Aerobic Bacilli*

To understand which features of the nutrition of *B. stearothermophilus* contributed to its thermophily, knowledge of less thermophilic species and of mesophiles is essential. Most members of the *Bacillus coagulans* group grow between 33° and 55° C. Many of them grow at 60° C.[3] Preliminary experiments with 23 strains of *B. coagulans* (obtained through the kindness of Doctor Carl S. Pederson) grew well in the basal medium developed for the B_{12}-methionine strain of *B. stearothermophilus*. In agreement with the findings of Knight and Proom[11] and of Campbell and Williams,[5] most members of this species required both vitamins and amino acids for dense growth. One of these, No. 43P, responded to PGA when combined with cyanocobalamin or methionine. Campbell and Williams described a strain of *B. coagulans* that also required histidine and folic acid at 36° to 55° C. Three other strains showed additional requirements at higher temperatures (TABLE 7). The nutritional patterns of other strains are now being investigated.

Most members of the mesophilic species *B. circulans* grew between

TABLE 6

BASAL MEDIUM FOR *B. STEAROTHERMOPHILUS* NO. 3084 (FOLIC STRAIN)[38]

Versen-Ol	0.004 g.	DL-Aspartic acid	0.05 g.
"Fe^3-specific"	0.01 g.	DL-Alanine	0.05 g.
K glycerophosphate	0.05 g.	Glycine	0.05 g.
$MgSO_4 \cdot 7H_2O$	0.05 g.	L-Glutamic acid	0.08 g.
Aconitic acid	0.3 g.	DL-Methionine	5.0 mg.
Na acetate$\cdot 3H_2O$	0.04 g.	DL-Threonine	10 mg.
Sucrose	1.5 g.	Mo (as NH_4 salt)	0.1 mg.
DL-Asparagine	0.25 g.	V (as SO_4)	0.01 mg.
"Metals 44"	1.5 ml.	Thiamine HCl	0.2 mg.
"DED"	0.5 g.	Pyridoxamine $\cdot$ 2 HCl	0.02 mg.
Ca (as Cl)	2.0 mg.	Biotin	1.0 μg.

Distilled water to 100 ml.; pH 6.9-7.1

Notes:

1. *Metals.* This strain appears to require a different balance of metal ions. "Fe^3-specific" (N, N-dihydroxyethylglycine, obtained from Versenes, Inc.) was supplied together with Versen-Ol because of the increased concentration of iron in the medium (in "Metals 44," see TABLE 8).

2. *"DED"* = dihydroxyethylethylenediamine (gift of Carbide and Carbon Chemicals, Inc.) was used instead of 2-amino(2-aminoethyl) ethanol because of its lighter color. Unfortunately it is no longer available.

3. *Pyridoxamine* was required at 55° C. but not at 45° C. *Thiamine* and *biotin* were absolute requirements.

4. *Amino acids* were stimulatory, not essential.

28° C. (or lower) to 37° to 45° C.[3] A survey of 54 strains obtained from Doctor Ruth E. Gordon showed that all required thiamine and biotin, in conformity with preliminary results of Knight and Proom.[11] As mentioned in our previous paper,[6] some strains also require riboflavin and reduced sulfur.

Discussion

1. *Bacilli and the General Theory of Thermophily*

The explanation of thermophily is proving not to be simple. It has been repeatedly shown that bacilli contain unusually stable enzymes. For example, Stewart and Taylor[12] found that alanine racemase extracted from spores of *B. terminalis* remained active after exposure to 80° C. for 2 hours. The corresponding enzyme from vegetative cells was almost completely inactivated in 15 minutes. In continuation of the studies of the Nebraska group on thermophily and "thermal enzymes," Militzer and Burns[13] found that while pyruvic oxidase from *B. stearothermophilus* was quickly inactivated at 60° to 65° C., malic dehydrogenase, cytochrome oxidase and aconitase were stable (see reviews by Allen[1] and by Clegg and Jacobs[2] for references to earlier work)

The comparatively high concentration of growth factors required by thermophiles noted by previous workers[4] and by ourselves nevertheless implies that the metabolism of thermophiles may be characterized by an accelerated wearing out of catalytic metabolites such as growth factors. Perhaps this is in keeping with their enormous consumption of substrate. The need shown by thermophiles for high concentrations of growth factors appears to support the "dynamic" theory of thermophily advanced by Allen. Unfortunately, there are almost no quantitative data on nutritional

Table 7

Effect of Incubation Temperature on the Nutritional Requirements of *B. coagulans*
After Campbell and Williams[5]

Strain	*36° C.*	*45° C.*	*55° C.*
12	Histidine, leucine, folic, valine	Histidine, leucine, folic	Same as 45° C.
32	Methionine, tryptophan	Methionine, tryptophan, histidine, folic	Same as 45° C.
1039	Folic	Folic	Folic, histidine, methionine, nicotinic

requirements as a function of temperature. Such information, considered together with the results of experiments on the rate of incorporation of isotopically labeled nutrients, might allow calculation of enzyme turnover numbers as a function of temperature, and so provide a critical examination of the value of thermostability in increasing the efficiency of enzymes at elevated temperatures.

The recent studies of Militzer and Burns [13] indicate that the dynamic and the thermostable-enzyme theories of thermophily are not mutually exclusive. The instability of the pyruvic oxidase from *B. stearothermophilus* was lessened in the presence of Mg, pyruvate, and O_2. Since the supply of pyruvate—an unstable compound—has to be maintained by metabolic activity, the stability of pyruvate oxidase would depend on an accelerated formation of pyruvate.

There are indications that fatty acids have a special role in thermophily. For example, when *Clostridium botulinum* was grown in 0.001 *M* palmitate or oleate, the spores had a greatly increased thermostability. [14] It is tempting to speculate that a similar reaction may play a part in the ability of oleate, palmitate myristate, and stearate partly to overcome the growth retardation of mice whose metabolism had been accelerated by the administration of thyroactive substances. [15, 16] Little is known of the fatty acid content of thermophiles as compared with mesophiles. Dyer [17] found that the lipids of *B. stearothermophilus* were of the sphingomyelin rather than the lecithin type. Sphingomyelins are more stable to hydrolysis than are lecithins.

For several reasons, the present nutritional work cannot present a fair picture of the participation of lipids in thermophily. For one, incubation temperatures were not pushed above 80° C. It might be expected that, at this and possibly at higher temperatures, the increased miscibility of lipids in the aqueous phase would greatly increase their availability and thus allow a better assessment of their worth. Also, the strains used had been isolated on peptone media and the like, which are virtually lipid-free. The present collection might therefore have been selected for forms which are largely indifferent to fat-soluble, water-insoluble nutrients, at least at incubation temperatures below 80° C. It is encouraging that so large a measure of tolerance to bile was encountered. The inhibitory material in bile whose action, as described earlier, was counteracted by supplements of isoleucine and other amino acids, has not been identified For studies of lipid nutrition it would be desirable to replace whole bile by purified bile acids or equivalent solubilizing agents.

The thermophiles studied here may be unrepresentative in another way: they were isolated on autoclaved media—a procedure which might

have selected against strains having an enhanced ability to utilize thermolabile nutrients. The recent studies of Atwood and Mukai[18] in which the proportion of *Neurospora* mutants that had lost indispensable functions ("indispenable" functions are defined as biosyntheses which cannot be compensated for by the use of complex media) was greater when tested by the heterocaryon method than by the temperature method. That is to say, many mutants could be maintained as heterocaryons but not as homocaryons. There was a smaller proportion of mutants that could not grow at 33° C. with the aid of supplements, but grew at lower temperatures without the supplements. The more efficient recovery of mutants by the heterocaryon method hints that the transfer of nutrients from one cytoplasm to another is far more efficient that the transfer of nutrients into the cell from an artificial aqueous medium. The nutrients that present difficulties when they are to be supplied exogenously are those which are either thermolabile, high-molecular, or fat-soluble

The increased complexity of nutritional requirements generally met at higher temperatures may reflect inabilities of syntheses to keep ahead of destruction. The thermophiles studied here can utilize a great variety of metabolites, some of rather high molecular weight, *e.g.*, folic acid and cyanocobalamin. Furthermore, the thermophiles utilize, without obvious inhibition, nutrients that are supplied in high initial concentration. This points to their possessing a high permeability and osmotic tolerance so that the uptake of building blocks and catalysts is rapid enough to keep the synthetic apparatus fully operative.

Judged from the kind of nutritional supplements required to support growth at elevated temperatures, the heat-induced synthetic disabilities of thermophilic bacilli are not sharply different from those observed in other organisms. For example, a strain of *Escherichia coli*, when grown at 44° C., had a special requirement for glutamic acid or nicotinic acid.[19] *Pasteurella pestis*, grown at 36° C. instead of 28° C., required a supplement of alanine, leucine, serine, threonine biotin, and pantothenate.[20] *Coprinus fimetarius* required methionine at 35 to 40° C., but not at 25° C.[21] Enhanced needs for low-molecular building blocks are surmised from the report that if *E. coli* is "sterilized" by heating at 72° C. the cells may be reactivated by suspension in a mixture of malate, succinate, *cis*-aconitate, and related compounds.[22]

In a few instances, in "temperature" mutants, the site of the synthetic blocks has been identified as an alteration in the heat stability of an enzyme. Maas and Davis[23] found that a *Neurospora* mutant that required pantothenate for growth above 30° C. had an excessively heat-labile pantothenate-synthesizing enzyme, and Horowitz and Fling[24] described a series of *Neurospora* mutants with variously thermostable tyrosinases controlled by Mendelian factors.

2. *Use of strains of* B. stearothermophilus *as assay organisms*

The rapid growth of strains of *B. stearothermophilus* in the chemically defined media described here suggests their use for asaying the growth factors needed by particular strains. As briefly sketched elsewhere,[7] they appear, as a group, to have important advantages:

(a) *Elimination of heat sterilization.* We have observed that experimental media incubated at 55° C. or higher appear free from significant contamination. To provide a more rigorous test, sterile agar plates with media suitable for thermophiles were exposed as long as 24 hours in the laboratory, then incubated at 37°, 55°, and 65° C. Many colonies overgrew the 37° C. plates; there were no colonies in the 55° C. and 65° C. plates. Control plates seeded with thermophiles showed little or no growth at 37° C., and heavy growth at 55° C. and 65° C. Evidently, airborne thermophiles are not a serious hazard to sterility. Obviation of autoclaving would permit the convenient assay of thermolabile materials, both water- and fat-soluble. The bioautograph method of assaying paper chromatographs would be especially easy to apply here, since it is difficult to run chromatograms aseptically.

(b) *Ease of maintenance and simplicity of culture media.* The chemically defined basal media developed in this study require fewer constituents than do media for the streptococci and lactobacilli commonly employed as assay organisms. As mentioned earlier, in common with other aerobic sporeformers, they may be stored for long periods in the cold with little decline in viability. Since most strains of *B. stearothermophilus* do not grow below 37° C., storage at room temperature may prove to be as effective a conservation measure as storage in a freezer would be for mesophiles. Finally, their good growth on agar surfaces recommends them for pad assays as well as for bioautography.

3. *Specific Nutritional Patterns of* B. stearothermophilus

(a) *B_{12}-methionine relations.* Superficially, the interchangeability of cyanocobalamin and methionine for strain 1503 resembles that described for mutant 113-3 of *E. coli*,[8] which is now widely employed as an assay organism. It remains to be seen whether the biosynthetic block in *B. stearothermophilus* is situated in the "red" part of the cobalamin molecule, as it is for the *E. coli* mutant.[7]

(b) *B_{12}-folic-PAB relations.* Strain 3084 appears to be the first microorganism in which the connection between folic acid and B_{12} has been demonstrated without the use of inhibitors. The general pattern appears to be essentially that worked out by Shive *et al.*[25] in reversing sulfanilamide inhibition of wild-type *E. coli*. The interchangeability of PAB, PGA, and folinic acid, and the inertness of pteroic acid, resembled

the pattern described by Lascelles *et al.*[10] for *Streptococcus equinus* P60. The PAB-folic strain, No. 3084, is now being used at Mt. Sinai Hospital in studies of folic acid metabolism in a variety of clinical problems.

Amino acid interactions. The need to supply leucine, isoleucine, and valine in rather narrowly limited proportions is familiar in microbial nutrition. A similar situation prevails in *Leuconostoc dextranicum* 8086[26] and in *Pasteurella pestis.*[20]

Miscellaneous requirements. The specificity of the biotin requirement in several strains of *B. stearothermophilus* and *B. coagulans* was studied by Campbell and Williams.[27] Some strains of *B. stearothermophilus* used either aspartate or oleate in place of biotin. The "PAB-folic" strain, No. 3084, included in their studies, utilized oleate at 45° and 55° C., but not at 36° C. The biotin requirement of some strains was not bypassed by oleate and aspartate, or by tricarboxylic acid cycle intermediates. This suggests that certain strains of *B. stearothermophilus* may be suitable assay organisms for biotin. The specificity of the thiamine and nicotinic acid requirements has not been investigated.

4. *Some Problems and Prospects*

As emphasized earlier, the strains used in the present study may be but a narrow sampling of the forms existing in nature. Enrichment cultures with lipid substrates at elevated temperatures, with or without emulsifying agents, might yield strains exhibiting requirements for fat-soluble factors. One may envisage their use as assay organisms for fat-soluble factors. Such assays might allow the precise localization of fat-soluble factors in tissue slices, the slices being used for bioautographs or, better, for histograms, much as paper chromatograms are "developed" microbiologically in the bioautograph technique. The poor diffusibility of fat-soluble factors makes them especially fit for such studies.

The present study indicates that thermophiles require high concentrations of nutrients, so that the culture media exerted rather high osmotic pressures. Were thermophiles not osmoresistant, their growth at elevated temperatures would be curtailed. Our impression, derived from the later experiments of this investigation in which concentrated media were used, was that we had not come near the osmotic limits for growth. A recently described medium for *B. subtilis* contains as major constituents, glycerol, 8.0 per cent; glutamic acid, 1.2 per cent; citric acid, 1.2 per cent; and NH_4Cl, 0.7 per cent—a medium at least twice as concentrated as any we have used. It is tempting to speculate that the widespread osmotolerance of sporeformers in general, coupled with their high permeability, favored the development of thermophily in this group.

The abundant growth obtained in this study of some strains of *B. stearothermophilus*, at temperatures as high as 80° C. in chemically at temperatures as high as 80° C. in chemically defined media, underscores the suitability of thermophilic bacilli for studies of the stability of cell constituents.

Although recent studies have chosen to lay stress on the conditions for growth at elevated temperatures, it must be recognized that the failure of many thermophiles to grow below 40°, or even below 45° C., is a mystery to which, at present, there are fewer clues than there are to high-temperature growth. Laboratory studies along these lines may help resolve such an apparent paradox as the occurrence of "obligately" thermophilic bacilli at the ocean bottom.[29] Ecological aspects of the vitamin requirements of mesophilic and thermophilic bacilli will be discussed in a later communication.

Part II. Temperature Tolerance and Substrate Utilization in *Euglena gracilis*

Introduction

Some strains of *Euglena gracilis* grow well at temperatures lethal to other strains. In preliminary experiments that were incidental to attempts to improve the *Euglena* assay for vitamin B_{12}, it was observed that the "high temperature" strains used a wider range of organic substrates than did the "low temperature" strains. This difference seemed to go along with susceptibility to permanent destruction of chloroplasts ("bleaching") by heat or by streptomycin. The *E. gracilis* species complex appeared, then, to be favorable objects for investigating why some members of a well-defined taxonomic group may grow at temperatures which kill the others.

The physiology of the euglenoid flagellates has been reviewed in detail[30,31] and so only immediately pertinent details will be mentioned.

Heat- and Streptomycin-Induced Bleaching

After the discovery that streptomycin induced a permanent loss of chloroplasts in two strains of *E. gracilis* (vars. *bacillaris* and *urophora*[32]), it was observed that the *bacillaris* strain was also susceptible to loss of its chloroplasts by exposure to temperatures which were inhibitory but not lethal. This phenomenon was investigated in detail by the Pringsheims.[33]

The 26 strains studied by them fell into three groups:

(1) Strains not bleached at all. The Mainx strain, which does not grow above 28° C., was in this class.

(2 and 3) Strains that had a higher optimum temperature for growth.

(3) Strains in which bleaching was possible. The best temperature for complete bleaching was 34° to 35° C., which is just above the optimum temperature for growth.

Susceptibility to bleaching by heat and by streptomycin ran parallel.

Growth of *E. gracilis* at elevated temperatures (elevated, that is, for an alga) with consequent loss of photosynthesis, imposes synthetic disabilities—a situation paralleling the additional exogenous requirements found in thermophilic bacilli grown at elevated temperatures (PART I.). If the equivalent of the products of these blocked syntheses (for *Euglena*, products of photosynthesis) can be supplied exogenously, then bleached *Euglenas* will survive. It is not unlikely that growth at elevated temperatures consumes essential metabolites faster than they are replenished by syntheses that are powered by photosynthesis.

When grown in the dark, *E. gracilis* loses its chloroplasts. Such cultures become fully green when illuminated.

A culture medium that will keep cultures green in the dark has not been found. It was thought that a re-examination of this problem might increase understanding of the hypothetical biosynthetic block underlying permanent bleaching by heat (and streptomycin). There was also the hope that an exploration of the upper temperature limit for growth and for maintenance of the photosynthetic apparatus, when compared with the patterns in thermophilic bacilli of nutritional requirements as a function of temperature, might reveal which biosynthetic systems were especially vulnerable to heat inactivation. This expectation was not fulfilled, but the experiments showed that, within the *E. gracilis* group, there existed sharper differences than had been supposed in respect to temperature optima for growth, and that at leaxt one strain, "Z", grew even more vigorously than did the *bacillaris* strain upon a considerable variety of organic substrates. The "Z" strain, therefore, may be especially favorable for studying temperature phenomena in *Euglena*.

The present study was also designed to test in more detail the hypothesis that those strains of *E. gracilis* that have comparatively high optimal temperature for growth also have the greater ability to utilize exogenous substrates.

Culture Methods

The culture methods were the same as those used in previous studies of photosynthetic organisms.[34] Media were distributed in 5-ml. amounts in 10- or 25- ml. glass-capped micro-Fernbach flasks (Kimble Catalog No. 26020-s-43). The larger surface: volume ratio permitted by these flasks was desirable because of the lessened internal shading.

Growth was expressed as optical density as measured with a Welch Densichron. Because of the large light-scattering error caused by the

size of the cells and their refractile paramylum granules, cultures to be scored were diluted as much as 1:200, since the scattering error was less in dilute suspensions.

Culture Media

The media were modifications of those undergoing study for the *Euglena* assay for vitamin B_{12}. They were originally devised for such strains as *bacillaris* and "Z", which vigorously utilize organic substrates and attain exceptionally high densities (O.D.'s > 10).

The concentrated medium used in earlier experiments (TABLE 8) was used for initial screening of strains of *E. gracilis* into "Mainx-type" (low temperature) and "*bacillaris*-type" groups (TABLE 9).

Cultures

With the exception of the *bacillaris* strain (now available from the American Type Culture Collection and from the Culture Collection of Algae of the Department of Botany, Indiana University, Bloomington, Ind.), the strains used were obtained from the Collection of Protozoa and Algae of Cambridge University, Cambridge, England.

TABLE 8

CONCENTRATED LOW-PH MEDIUM FOR *EUGLENA GRACILIS*

KH_2PO_4	0.04 g.	"Metals 44"	0.4 ml.
DI-Malic acid	0.15 g.	Mo (as NH_4 salt)	0.02 mg.
$MgSO_4 \cdot 7H_2O$	0.05 g.	V (as SO_4)	0.01 mg.
$CaCO_3$	0.01 g.	Thiamine HCl	0.06 mg.
L-Glutamic acid	0.15 g.	B_{12}	0.4 μg.
DL-Aspartic acid	0.15 g.	Distilled water to 100 ml.; pH 3.4-3.6	
Sucrose	2.5 g.		
Glycine	0.06 g.		
NH_4HCO_3	0.06 g.		

Notes:

1. *Sucrose* is utilized in the presence of CO_2 or of compounds which are either constituents of, or are readily convertible into, tricarboxylic acid cycle intermediates; in the present medium this function is shared by the glutamic, aspartic, and malic acids. These interactions will be detailed in a later communication.

2. *Nitrogen sources.* Utilization of sucrose also depends on readily available N. The multiple N-sources in the medium allow denser growth than do single N-sources.

3. "Metals 44" has the following composition (mg./ml.):

EDTA	2.5	Cu	0.1
Zn	2.5	Co	0.05
Fe	1.0	B	0.02
Mn	0.5		

Results

Trial of 24 strains of *E. gracilis* using concentrated basal media (TABLE 8) revealed that the strains fell into two sharply distinct groups: (1) the Mainx and the *bacillaris*; and (2) the *bacillaris* type, with such strains as *O* in between.

All the cultures which grew at 34° C. proved to be permanently bleached when transferred in light. Cultures which had shown slight growth at 37° C. became green or yellow-green in light. Cultures showing fairly good growth (*e.g.* the *Z* and *bacillaris* strains) remained white.

Table 9

TEMPERATURE TRIALS ON 24 STRAINS OF *EUGLENA GRACILIS*
Basal Medium as in TABLE 8.

MEDIUM	*STRAINS*							
	*D***	*K****	*L*	*O*	*R*	*T*	*Z*	*bac.*
Light - 30° C.	0.14	0.04	0.16	10.0	10.0	12.0	12.0	12.0
Dark - 34° C.	0.08	0.05	0.1	0.42	0.8	9.0	16.0	9.0
Dark - 37° C.	0	0	0	0	1.0	1.5	6.0	1.5
C.S.* - 34° C., light	0.1	0.13	0.2	5.8	5.8	19.0	20.0	19.0
C.S. - 37° C., dark	0	0.06	0	0	1.0	1.9	4.2	1.9
C.S. + Trypticase 0.25% + ox bile 0.01% - 34° C., dark	0.12	0.6	0.2	2.7	5.8	15.0	30.0	15.0
As above, but 37° C.	0	0	0.13	0	2.0	0.6	1.9	0.6

*"C.S." = "complete supplement" (see "*Notes*," TABLE 4). It was used at 0.8 the standard concentration.

Strains *B, C, E, M, V, X, Y,* 26, and the Mainx strain did not grow in these media but grew in dilute peptone-semisolid agar control tubes, having the following composition:

K_2HPO_4	0.02 g.	Fe	0.1 mg.
Na_3 citrate·$2H_2O$	0.002 g.	Thiamine HCl	0.02 mg.
$MgSO_4 \cdot 7H_2O$	0.002 g.	Cyanocobalamin	0.05 μg.
Trypticase	0.05 g.	Agar	0.25 g.
Yeast autolysate	0.006 g.	Dist. water to 100 ml.; pH 6.2 to 6.6	

**Strains *F, G, H, I,* and *W* behaved like *D*.
***Strains *N* and *W* behaved like *K*.

The results support the conclusions of the Pringsheims and of Robbins *et al.*[35] that, for bleaching, the heat treatment had to be such that growth of the organism occurred.

It is seen from TABLE 8 that the supplements to the basal medium were ineffective in raising the upper temperature limit for growth. Many kinds of culture media were then tested with the *Z* strain either for their ability to maintain chlorophyll in the dark or for their specific ability to support growth at elevated temperatures. Particular attention was devoted to hematin, which has been identified as a precursor of chlorophyll,[36] and to bile as a source of fatty factors. It was recognized that while white cells contained hematin, artificially bleached cells might have hematin synthesis so impaired as to leave no surplus for chlorophyll

TABLE 10

USE OF SUBSTRATES BY THE *Z* AND MAINX STRAINS OF *E. GRACILIS*

Substrate g.%		*Z*		*Mainx*	
			Na acetate $\cdot 3H_2O$ *0.1%*		*Na acetate* $\cdot 3H_2O$ *0.1%*
None		0.26	0.90	0.06	0.28
Na acetate$\cdot 3H_2O$	0.05	0.80	1.24	0.2	0.37
Dl-Malate	0.05	0.42	0.42	0.08	0.12
" "	0.25	0.48	0.33	0.04	0.09
L-Glutamate	0.05	0.64	0.53	0.02	0.14
" "	0.25	0.8	0.74	0.06	0.12
DL-Aspartate	0.05	0.43	0.55	0.07	0.16
" "	0.2	1.70	0.48	0.21	0.20
Glycine	0.05	0.86	1.70	0.20	0.20
"	0.25	0.96	1.00	0.14	0.26

Notes:
The basal medium had the following composition:

K_2PO_4	0.02 g.	"Metals 44"	0.1 ml.
Nitrilotriacetic acid	0.01 g.	Thiamine HCl	0.05 μg.
$MgSO_4 \cdot 7H_2O$	0.05 g.	Cyanocobalamin	0.5 μg.
$(NH_4)_2 SO_4$	0.04 g.	Distilled water to 100 ml.;	
Ca (as Cl^-)	1.0 mg.	pH 6.0 to 6.5	

The nitrilotriacetic acid served as a metal binder. It was used in this later work because it is commercially available as the free acid, a feature that is especially desirable for work with osmotically intolerant organisms, as ballasting of the medium with excess Na ion is avoided. The solubility of the Ca and Mg complexes compares favorably with that of N-hydroxyethylethylenediamine triacetic acid, which is not yet available as the free acid.

synthesis. To insure the availability of hematin, it was used in alkaline media (pH 8.0 to 8.3) under conditions where it was known to be available to Trypanosomidae and to a staphylococcus with requirements for exogenous hematin.[37] Concentrations of ox bile as high as 40 mg. per cent were used in combination with hematin, "complete supplement," yeast autolysate, peptones, and the like.

Utilization of Substrates

As mentioned in the *Introduction*, it was of interest to determine whether the ability to grow at elevated temperatures correlates with versatility in use of substrates. The striking difference in this respect between the *Z* and Mainx strain is brought out in TABLE 10; acetate here served as the positive control. An extension of this type of experiment showed that the *bacillaris* strain was almost identical with *Z*, and the *B*, *C*, *D*, and *N* strains with the Mainx strain and with a strain of *E. viridis*. None of the Mainx-type strains grew in the dark on combinations of glutamate, aspartate, and malate; *Z* and *bacillaris* grew in the dark; all strains grew well in the dark in the acetate controls.

The utilization of sugars, another index of permeability (see *"Notes,"* TABLE 8), allowed an especially sharp distinction between the "high temperature" and "low temperature" strains (TABLE 11). Acid media were used to favor penetration of the amino acids.

Osmotic Tolerances

The "high temperature" strains, tested in a dilute version of the "concentrated" medium previously described (TABLE 8), were clearly more osmoresistant than the "low temperature" strains; the entire collection was used in these experiments. None of the "low temperature" strains grew in the medium supplemented with NaCl 1.2 per cent. Growth of the *R*, *Z*, and *T* strains, on the other hand, although slowed, was appreciable. The *Z* strain as usual made the best growth, the *bacillaris* gave just-visible growth. Results with pentaerythritol, chosen as a metabolically inert, nonionic solute, were similar.

Lower Temperature Limits for Growth

For routine maintenance of cultures, it was the practice to incubate stock cultures in dilute peptone media, *e.g.*, that described in TABLE 9. At 18° C., all the strains grew well. The four "high temperature" strains grew as well as the others under these conditions, and also grew well in the concentrated medium. When the temperature was lowered to 14° to 15° C., strains grew neither in the maintenance media nor in the concentrated media. The growth of the "low temperature" strains was slowed but little.

Discussion of *Euglena* Results

In the *Euglena* line, as in sporeformers, ability to grow at elevated temperatures seems to demand, among other things, a large capacity for utilizing exogenous nutrients. The high-temperature strains of both groups may pay a price for this ability. So far as examined, they have shown a diminished ability to grow at low temperatures. Why this should be is unknown. The occasional occurrence of exogenous requirements at lower temperatures is perhaps a useful clue (see DISCUSSION, PART I).

The greater osmotic tolerance of the "high temperature" *Euglenas* may be an effect of, rather than a contributing cause of, thermophily. Heightened metabolism of these *Euglenas* may do no more than exploit the latent capacity of their osmotic pump. For the "low temperature" strains to be validly compared with the "high temperature" strains, they should be grown under conditions of optimal generation of energy,

Table 11

Utilization of Sugars by "High Temperature" and "Low Temperature" Strains of *E. gracilis*

Supplements g.%	"High temperature" strains				"Low temperature" strains		
	Z	*bac.*	*R*	*T*	*B*	*C*	*D*
None	0.43	0.54	0.17	0.51	0.04	0.01	0.09
DL-Aspartate 0.1	0.25	0.41	0.17	0.25	0.15	0.37	0.07
DL-Asparagine 0.1	0.89	1.09	0.78	0.72	0.08	0.22	0.12
Glucose 1.0	1.92	1.42	10.0	10.0	0.03	0.03	0.05
Sucrose 1.5	0.26	4.0	12.0	10.0	0.02	0	0.04
Sucrose 1.5 + DL-Asparagine 0.1	1.45	10.0	18.0	20.0	0.03	0.02	0.03
Sucrose 1.5 + DL-Aspartate 0.1	1.20	1.54	18.0	20.0	0.06	0.04	0.04

Notes:

The basal medium used has the following composition:

KH_2PO_4	0.02 g.	"Metals 44"	0.1 ml.
$Mg{\cdot}SO_4{\cdot}7H_2O$	0.04 g.	Mo (as NH_4 salt)	0.01 mg.
$CaCO_3$	0.005 g.	V (as SO_4)	0.005 mg.
NH_4HCO_3	0.1 g.	Thiamine HCl	0.05 mg.
Pyromellitic acid	0.1 g.	Cyancobalamin	0.4 μg.

Distilled water to 100 ml., pH 3.2 to 3.6

Pyromellitic acid (gift of E. I. Du Pont de Nemours & Co., Bloomington, Del.), served as pH buffer. As a tetracarboxy acid, it was an efficient buffer while not contributing much to the osmotic pressure of the medium. The Mainx strain grew in 0.4% of the acid, at pH 3.1, without inhibition.

i.e., their photosynthetic apparatus must be made to operate at capacity by provision of extra light and CO_2. It will be interesting to see whether their temperature optima are thereby raised.

The repeated failure to find nutrients specifically able (1) to prevent decolorization in darkness, (2) to prevent irreversible bleaching at temperatures above the optima for growth, and (3) to raise the upper temperature limit for growth, emphasizes how formidable is the problem of identifying, in *Euglena*, the temperature-sensitive biosynthesis systems concerned in the maintenance of the photosynthetic apparatus and of essential functions. Indeed, the goal of growing *Euglenas* at 40° C. now seems farther away than that of growing thermophilic bacilli at 85° C.

General Summary (Parts I and II)

Chemically-defined culture media were devised for several strains of *Bacillus stearothermophilus* and *B. coagulans*. One strain of *B. stearothermophilus* was grown at 80° C. in chemically defined media supplemented with methionine, leucine, and phenylalanine.

The *p*-aminobenzoic (PAB)-folic acid requirement of one strain of *B. stearothermophilus* was bypassed by a mixture of xanthine, thymine, and high concentrations of cyanocobalamin. This mixture, however, did not allow full growth. Similar results with this strain were obtained in sulfanilamide inhibition studies. Folinic acid (Leucovorin) but not pteroic acid satisfied the PAB-folic requirement.

Another strain responded interchangeably to cyanocobalamin and methionine. Dense growth at 55° to 65° C., in the presence of 40 mg. per cent whole dried bile, was permitted by supplementation with certain proportions of isoleucine, valine, and leucine.

Use of thermophilic (*i.e.*, able to grow at 55° C. or higher) bacilli as assay organisms was considered to be promising in view of the freedom from contamination of cultures incubated at 55° C. or higher. Their tolerance of such effective fat-solubilizers as bile, coupled with their high permeability for both low- and high-molecular metabolites, was construed as favoring their potential use for assaying fat-soluble and thermolabile metabolites, for which few satisfactory microbiological assays are now available. Possible limitations in current isolation methods in respect to strains requiring fat-soluble or thermolabile nutrients were pointed out.

Thermophily was considered to depend not only on the presence of unusually thermostable enzymes and on the functioning of greatly expanded biosynthetic systems. The relatively high permeability and high osmotic tolerances of thermophiles permit the efficient provision to these systems of ample supplies of catalysts, fuel, and building blocks.

"High temperature" strains of the phytoflagellate *Euglena gracilis* utilize a wider range of substrates than do "low temperature" strains, and have a somewhat higher osmotic tolerance. These findings support the idea that a heightened permeability is one of the prerequisites for thermophily.

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